1

UNCOUPLING AGENTS

This invention relates to uncoupling agents for use in the control of bacterial biomass in aqueous systems, to the use of such agents and to a method of using such agents.

Mechanistically, the generation of biomass in wastewater treatment arises from the consumption of nutrients in the wastewater. By a process of respiration, the nutrients are oxidised and this releases energy, which can be used by the microorganisms in cell division. If this energy could be "wasted", this would result in a reduced generation of biomass by inhibition of energy production

Bacterial biomass produced during the treatment of wastewater is costly to dispose of and so a reduction in biomass leads to reduced disposal costs.

Details of the biochemistry and mechanisms involved in cell respiration are given in, for example, "Biochemistry", 3rd edition, author: Lubert Stryer, publisher: W.H. Freemen & Company, New York 1988. Also in "General Microbiology", 3rd edition, authors: Roger Y. Stanier, Michael Doudoroff and Edward A. Adelberg, publisher: Macmillan, 1971. For the purposes of this patent application, the important point to note is that consumption of nutrients causes a proton flux which, in another part of the cell, is used to create adenosine triphosphate (ATP), via oxidative phosphorylation. ATP provides the energy for cell processes including cell division.

Hitherto, certain organic chemical compounds have been identified in the laboratory that will act as "uncoupling agents" to disrupt the proton flux. This results in a reduction in bacterial cell division by uncoupling the

2

proton flux thus reducing the energy available to the bacteria. The wasted energy appears as heat. The use of uncoupling agents can often cause increased nutrient consumption (which is desirable) due to the loss of respiratory control. However, the aforementioned uncoupling agents

5   are usually phenolic compounds, e.g. 2,4-dinitrophenol and para-nitrophenol; halogenated products, e.g. carbonylcyanide-p-trifluoromethoxyphenylhydrazone and 2,4,5-trichlorophenol or nitro compounds. All are unsuitable for use in aqueous systems due to their high environmental toxicity.

10

Accordingly the present invention provides the use of a water-soluble biocide as an uncoupling agent at an effective amount to control bacterial biomass in an aqueous system.
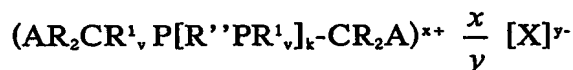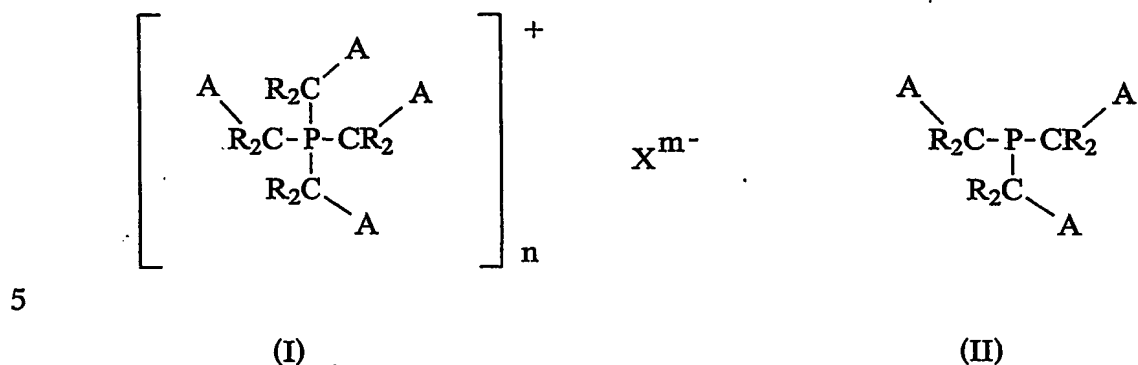
15   The effective amount of the water-soluble biocide added to the aqueous system is up to 5000mg/l, for example up to 3000mg/l, such as up to 1000mg/l. Preferably, the effective amount of the water-soluble biocide added to the aqueous system is from 0.005 mg/l to 500 mg/l, for example from 0.01mg/l to 300mg/l, such as from 0.05mg/l to 100mg/l. More

20   preferably, the effective amount of water-soluble biocide is from 0.1 to 10 mg/l, for example from 0.5mg/l to 7.5mg/l, such as from 1 to 5mg/l.

Alternatively, the effective amount of said water-soluble biocide may be from 0.1 to 10,000 milligrams per gram of sludge solids in the aqueous

25   system. Preferably, from 0.5 to 1000mg/g, for example, from 1 to 500mg/g, such as from 5 to 100mg/g.

In a preferred embodiment the current invention provides the use of a water-soluble biocide as an uncoupling agent, the water-soluble biocide

30   comprising an alkyl-substituted phosphonium compound of formula (I), an

alkyl-substituted phosphine of formula (II) and a condensate of formula (III):

$$\left[\begin{array}{c} A \\ A \diagdown R_2C \diagup A \\ R_2C-P-CR_2 \\ R_2C \diagdown A \end{array} \right]^{+}_{n} \quad X^{m-}$$

(I)

$$\begin{array}{c} A \diagdown \qquad \diagup A \\ R_2C-P-CR_2 \\ R_2C \diagdown A \end{array}$$

(II)

$$(AR_2CR^1{}_v P[R''PR^1{}_v]_k\text{-}CR_2A)^{x+} \dfrac{x}{y} [X]^{y-}$$

(III)

wherein:

X is an anion;

n is the valency of X represented by m;

each A can be the same or different and is selected from OH, OR, SO_3R, PO_3R_2, COOH, COOR, SO_3H, PO_3H_2, CH_2COOH, substituted alkyl, aryl and substituted amino groups;

each R, and each R, when present, in each A group, is independently selected from hydrogen, a C_1 to C_{20} alkyl, aryl, substituted alkyl or aryl, carboxy or carboxy ester; wherein each CR_2 group may be the same or different;

R'' is a divalent hydrocarbon radical having from 2-20 carbon atoms and is optionally substituted with one or more substituents selected from the group consisting of halogen, hydroxy, carboxy, amino, alkylamino, or $PR^1_mCH_2OH$ groups or interrupted by one or more ether or carbonyl
5   linkages;

each R^1 is independently a monovalent hydrocarbon radical having from 1 to 25 carbon atoms and optionally substituted with one or more substituents selected from the group consisting of halogen, hydroxy,
10   carboxy, amino, alkylamino, or $PR^1_vCH_2OH$ groups or interrupted by one or more ether or carbonyl linkages; and

in formula (III) each v is 1 or 2, k is from 0 to 10 (e.g. from 1 to 10), x is the number of groups in the molecule having v = 2 and X is a
15   compatible anion of valency y such that the compound is water-soluble.

X is preferably selected from the group consisting of chloride, sulphate, phosphate, acetate and bromide.

20   The alkyl-substituted phosphonium compound is preferably tetrakis (hydroxymethyl) phosphonium sulphate.   Alternatively, the alkyl-substituted phosphonium compound may be selected from the group consisting of tetrakis (hydroxymethyl) phosphonium chloride, tetrakis (hydroxymethyl) phosphonium bromide and tetrakis (hydroxymethyl)
25   phosphonium phosphate.

The   condensate   is   preferably   a   condensate   of tris(hydroxyorgano)phosphine with a nitrogen containing compound.

The nitrogen containing compound is preferably urea. Alternatively the nitrogen containing compound is selected from a C_{1-20} alkylamine, dicyandiamide, thiourea and guanidine

5   Alternatively the invention provides the use as an uncoupling agent a water-soluble biocide that comprises a compound selected from the following:

- quaternary ammonium compounds, e.g. dodecyl trimethyl
10   ammonium chloride, cetyltrimethylammmonium bromide, benzalkonium chloride, didecyldimethylammonium chloride and alkyldimethylbenzylammonium chloride;

- polymeric quaternary ammonium compounds, e.g. polyoxyethylene (dimethylimino) ethylene dichloride;

15 - polymeric biguanide hydrochlorides, e.g. polyhexamethylenebiguanide hydrochloride, dodecylguanidine hydrochloride;

- tris(hydroxymethyl)nitromethane;

- 4,4-dimethylozazolidine;

20 - phenoxypropanol;

- phenoxyethanol;

- glyoxal;

- acrolein;

- aldehydes, e.g. formaldehyde, glutaraldehyde;

25 - triazines, e.g. 1,3,5-tris(2-hydroxyethyl)-1,3,5-hexahydrotriazine;

- quaternary phosphonium compounds, e.g. tributyltetradecylphosphonium chloride and tetradecyl tributyl phosphonium chloride;

- 2-bromo-4-hydroxyacetophenone;

- carbamates, e.g. sodium N-dimethyldithiocarbamate, disodium ethylene bisdithiocarbamate;

- tertbuthylazine;

- tetrachloro-2,4,6-cyano-3-benzonitrile;

- thiazole and isothiazole derivatives such as 2-methyl-4-isothiazolin-3-one, 5-chloro-2-methyl-4-isothiazolin-3-one, 5-chloro-2-methyl-3(2H)-isothiazolone and 1,2-benzisothiazolin-3-one;

- compounds with activated halogen groups such as 2-bromo-2-nitro-propan-1,3-diol and 2,2-dibromo-3-nitrilopropionamide.

- bis chloromethyl sulphone, and

- methylene bis thiocyanate.

The water-soluble biocide for use as an uncoupling agent may be formulated with one or more of the following chemicals conventionally used in wastewater treatment:

a surfactant;
an antifoam;
a scale inhibitor;
a corrosion inhibitor;
a biocide;

a flocculant;

a dewatering aid, and

a dispersant.

5   Preferably, the aqueous system will be a wastewater treatment plant that
       is used for the treatment of industrial or municipal effluent. Such a plant
       typically takes in wastewater from industrial processes (e.g. paper
       production, food processing, chemical industry) and/or domestic and
       institutional habitations and the like and, by using micro-organisms in
10   aerobic, anoxic (e.g. denitrification) and/or anaerobic processes, to
       consume organic contaminants and render the water fit for re-use or
       discharge into the environment.

       The present invention also provides the use as an uncoupling agent of
15   conventional, water-soluble, water treatment biocides as defined above.

       The present invention further provides an uncoupling agent comprising
       one or more conventional water-soluble, water treatment biocide(s) as
       defined above.
20

       The present invention further provides a method for controlling the
       growth of bacterial biomass in an aqueous system comprising adding to,
       or contacting with, the aqueous system an effective amount of an
       uncoupling agent which is a water-soluble biocide as defined above.
25

       In a preferred embodiment the present invention provides a method for
       controlling the growth of bacterial biomass in an aqueous system, which
       method comprises contacting an effective amount of an uncoupling agent
       which is a water-soluble biocide as defined above directly with the
30   bacterial biomass.

8

The effective amount of the water-soluble biocide added to the aqueous system is up to 5000mg/l, for example up to 3000mg/l, such as up to 1000mg/l. Preferably, the effective amount of the water-soluble biocide added to the aqueous system is from 0.005 mg/l to 500 mg/l, for example

5   from 0.01mg/l to 300mg/l, such as from 0.05mg/l to 100mg/l. More preferably, the effective amount of water-soluble biocide is from 0.1 to 10 mg/l, for example from 0.5mg/l to 7.5mg/l, such as from 1 to 5mg/l. Alternatively, the effective amount of said water-soluble biocide may be from 0.1 to 10,000 milligrams per gram of sludge solids in the aqueous

10   system. Preferably, from 0.5 to 1000mg/g, for example, from 1 to 500mg/g, such as from 5 to 100mg/g.

Contacting the bacterial biomass with the water-soluble biocide directly has been found to lead to improved efficiency of the uncoupling agent in

15   controlling bacterial biomass. The direct contact of the water-soluble biocide with bacterial biomass is termed "flash dosing" or "flash mixing". It has been found that if the uncoupling agent is simply added directly to a bioreactor containing sludge then the efficacy of the agent is substantially reduced as the uncoupling agent is able to interact with other

20   matter in the bioreactor and the action of the water-soluble biocide is substantially reduced.

The invention will now be described merely by way of example with reference to the following examples and accompanying figures, of which:

25

Figure 1 is a diagram illustrating the process of flash mixing;

Figure 2 is a diagram of a semi-continuous chemostat reactor system used in the present invention;

30

9

Figure 3 is a diagram of an Oxitop® BOD system used to obtain oxygen consumption data, and

Figure 4 is a graph showing percentage uncoupling of four uncoupling
5   agents.

Referring to figure 1 a typical bioreactor B is shown with a recirculating line R into which the water-soluble biocide is injected. The bioreactor contains sludge, which is essentially a mass of typical wastewater
10   treatment microorganisms such as bacteria, protozoa, worms and fungi. The bioreactor B has an inlet I for wastewater and an outlet O for treated water. The sludge is extracted from the bioreactor into the recirculation line R by means of a pump P. Typically, a peristaltic pump is used. The candidate water-soluble biocide is introduced to the sludge in the
15   recirculating line via a needle connected to a syringe pump N. It will be appreciated that in a commercial scale bioreactor the water-soluble biocide will be introduced via a tee-junction or an injection quill and a progressive cavity pump may replace the peristaltic pump. This enables direct contact of the sludge with the water-soluble biocide. The water-
20   soluble biocide may be introduced into the recirculation line R at a location behind or ahead of the pump P.

However, it will be appreciated that the implementation of the invention is not restricted to using the method of "flash mixing" and other dosing
25   methods may be used if so desired.

Example 1 - Semi-Continuous Pilot Study with Flash Mixing

1. Equipment
30

A series of semi-continuous chemostat reactors was constructed to

simulate wastewater treatment processes. The arrangement of a semi-
continuous chemostat reactor is shown in figure 2.

5 The central part of the equipment is the biological chemostat reactor 1.
The reactor vessel 2 is made of glass, with an internal volume of 10 litres
and is fitted with pH 3 and dissolved oxygen 4 sensors and an air sparge
5. This contains the sludge, which is, essentially, a mass of typical
wastewater treatment microorganisms. The reactor operates at an ambient
temperature (approximately 20°C).

10

Simulated wastewater 13 is contained in a separate vessel 6 and
maintained at 4°C to prevent microbial spoilage. It is transferred into the
reactor via a supply line 7 and a peristaltic pump 8.

15 A recirculating line 9 is provided on the reactor comprising a length of
flexible silicone tubing and a peristaltic pump 10. The peristaltic pump
extracts the sludge from the reactor into the recirculation line. The
candidate water-soluble biocide is introduced into this line 9 through a
needle 11, which is fed by a syringe pump 12. Using this system it is
20 possible to achieve "flash mixing" which ensures rapid contact of the
water-soluble biocide with the sludge microorganisms. The internal
diameter of the recirculating line was 8 mm and the flow velocity was 0.5
metres per second, which gave a contact time, between candidate water-
soluble biocide and sludge of 3 seconds before it re-entered the reactor.

25

Treated effluent 17 is removed from the reactor into a collecting vessel 16
via an outlet line 15.

2. Procedure

30

11

The reactor was charged with sludge obtained from the Municipal wastewater treatment works at Courly - Lyon, in France. This sludge provided the "seed" to start the process off. The reactor was then filled, to the 5 litre mark, with simulated wastewater, adjusted to approximately

5   pH 7.5, and aeration was commenced. The reactor was continuously fed, at a rate of 800 ml per day, with simulated wastewater made to the recipe shown in Table B.

Each weekday (i.e. 5 times per week), 13.3% of the bioreactor contents

10   were withdrawn to maintain the equilibrium. This ensured a sludge age in the region of 7.5 days.

Also, each weekday, after the adjustments described in the preceding paragraph, candidate water-soluble biocide (as a dilute solution) was

15   injected into the system via the flash mixing recirculating line. The dilute solution was prepared by dissolving 10 grams of sodium hydrogen carbonate in approximately 900 ml of deionised water then adding 5 grams of candidate water-soluble biocide and mixing well. The mixture was then made up to 1 litre with deionised water. The appropriate

20   volume of this dilute solution was injected to achieve the required dose level.

The experiment was conducted for 52 days before decommissioning the system.

25

3. Test Concentrations

Compound X was evaluated in the test. This compound is a 75% aqueous solution of tetrakis(hydroxymethyl)phosphonium sulphate (THPS). Two

30   concentrations were evaluated: 2 and 4 milligrams per litre per day.

4. Results

Table A

Treatment	Specific Activity* (g COD/g MLSS.d)	Growth Yield (gMLSS/gCOD)	Growth Yield Reduction (%)
No Treatment (Control)	0.477	0.272	0
Compound X, 2 mg/l/day	0.582	0.202	27.7
Compound X, 4 mg/l/day	0.772	0.167	37.6

* Specific Activity is the rate of COD removal (grams per day) divided

5   by the quantity of sludge solids (grams) present in the reactor.

Also, the following observations were made during the pilot study:

- The control (untreated) study showed a proliferation of filamentous
10   bacteria in the sludge whereas these were substantially absent in
          sludge from Compound X treated systems.

- There was a significantly higher concentration of protozoans and
          other higher organisms in the treated sludge relative to the
15   untreated.

- Compound X treatment did not impair sludge flocculation.

5. Comments

20

This study confirmed that Compound X flash treatment, at low dose
levels, was capable of significantly reducing sludge growth whilst actually
increasing the specific activity of the sludge.

13

Also, sludge quality was significantly improved resulting in a sludge which would flocculate more easily (in case of filamentous bacteria proliferation), thus providing operational benefits.

5   **Simulated Wastewater Composition**

Table B

Component	Concentration (mg/l)
Acetic Acid	3400
Sucrose	600
Yeast Extract	1200
Casein Peptone	600
Potassium Sulphate	180
Magnesium Sulphate, heptahydrate	180
Ferrous Sulphate, heptahydrate	30
Calcium Chloride, anhydrous	20
Disodium Hydrogen	280
Sodium Hydrogen Carbonate	quantity to adjust to pH 4.3

Example 2 – Semi-Continuous Pilot Study with Pre-Dilution

10   **1. Equipment**

The equipment used in this test sequence was identical to that used for example 1 except that the reactor was not fitted with a recirculating line for flash mixing.

15

2. Procedure

The procedure was identical to that used for example 1 except that the method of dosing Compound X was different. In this case, 5 grams of
20   Compound X was mixed into a solution containing 5 grams of sodium hydrogen carbonate in 1 litre of deionised water.   4.0 ml of this solution was added to 500 ml of clarified wastewater which had been

14

extracted from the bioreactor. The treated clarified wastewater was then returned to the bioreactor and normal operation of the bioreactor was resumed. The final concentration of Compound X in the bioreactor therefore equates to 4 mg per litre. This procedure was

5   conducted daily, 5 times per week.

The experiment was conducted in parallel with example 1, i.e. for 52 days before decommissioning the system.

10   3. **Test Concentration**

A Compound X concentration of 4 milligrams per litre per day was used.

15   4. **Results**
     Table C

Treatment	Specific Activity* (g COD/gMLSS.d)	Growth Yield (gMLSS/gCOD)	Growth Yield Reduction (%)
No Treatment (Control)	0.477	0.272	0
Compound X, 4 mg/l/day	0.636	0.201	26.4

* Specific Activity is the rate of COD removal (grams per day) divided by the
   quantity of dry sludge solids (grams) present in the reactor.

20   5. **Comments**

The reduction in growth yield in this test was only 26.4% as compared to the 37.6% reduction (see example 1), at the same dose rate, when flash mixing was used. This demonstrates the benefit of flash mixing.

25

Example 3 – Uncoupling by Other Water Treatment Biocides

1. <u>Summary</u>

A laboratory screening test was developed to determine the impact of
chemical compounds on respiration parameters through an oxygen
consumption pattern analysis using a model based on published work by
Monod and Pirt[1]. Growth uncoupling is characterised by an increase in
oxygen consumption during bacterial growth in a synthetic growth
medium.

2. <u>Principle</u>

.Batch growth kinetic modelling and the interpretation of the oxygen
consumption kinetics were implemented by mathematical modelling.
Growth kinetics is described, according to Monod and Pirt's model, as
follows:

$$\frac{d\,[\,X\,]}{dt} = a \cdot \frac{d\,[\,S\,]}{dt} - b \cdot [\,X\,]$$

$$\frac{d\,[\,S\,]}{dt} = k \cdot [\,X\,] \cdot \frac{[\,S\,]}{K_s + [\,S\,]}$$

$$\frac{d\,[\,O_2\,]}{dt} = a \cdot \frac{d\,[\,S\,]}{dt} + b \cdot [\,X\,]$$

---

1.   Monod J. 1950, Ann Inst Pasteur, "La Technique de Culture Continue, Theorie et Applications", 79:390-410.   Monod J. 1942, "Recherches sur la Croissance des Cultures Bactériennes". Paris Herman p. 210. Pirt SJ. (1965) "The Maintenance Energy of Bacteria in Growing Cultures". Proceedings of the Royal Society of London B 163, 224-231. "Principles of Water Quality Management". Ed: W.W. Eckenfelder, Jr. CBI Publishing Company, Inc USA. 1980 : Chapter 9 - Biological Waste Treatment pp 249-275.

[X] : Concentration in biomass amount subject to endogenous metabolism, g MLSS/L

[S] : Carbon substrate concentration, g/L

d[X]/dt :   Biomass growth rate , g dry biomass/L.d

5 d[S]/dt :   Carbon substrate consumption rate, g /L.d

a :   Intrinsic substrate-biomass growth yield, g X/g substrate consumed

b :   Biomass maintenance coefficient, g dry biomass/g dry biomass. d

10 $d[O_2]$ /dt : Oxygen consumption rate, g O_2/L.d

a' :   Specific oxygen consumption coefficient, g O_2/g organic substrate consumed.

b' :   Endogenous respiration coefficient, g O_2/g X

k :   Maximum specific activity, g S/ g dry biomass.d

15 K_s :   Apparent substrate affinity coefficient or half maximum specific activity substrate concentration, mg/l   .

Relationship between a and a' : a' + (b'/b) * a = 1

Relationship between b and b' : b' = 1.25 * b

20

For the evaluation of proposed water-soluble biocides, the oxygen consumption kinetics are measured and, a process of computer iteration optimises the equation coefficients optimised to fit the oxygen consumption curve.

25

From the optimised coefficients, the degree of bacterial uncoupling can be calculated.

3. Equipment

An Oxitop® BOD System, supplied by Wissenschaftlich-Technische Werkstätten GmbH, Dr.-Karl-Slevost-Strasse 1, D-82362 Weilheim, Germany, was used for the screening tests (see figure 3). The complete system (see figure 3) comprised:

- 500 ml glass bottles 20 containing seeded culture medium 21;
- Each bottle was capped with a screw-on gas pressure meter 22 capable of recording up to 360 pressure data points. Sodium hydroxide pellets 23 were incorporated into the system to absorb carbon dioxide produced during bacterial respiration.
- The culture medium 21 was stirred using a magnetic stirrer 25 and a magnetic flea 24.
- The bottles were housed in a temperature controlled container (20°C)
- Data acquisition was by infra-red link with a hand-held control unit.
- Data was finally transferred from the hand-held control unit to an Excel° spreadsheet file for the calculations to be performed.

4. Test Biocides

Three conventional water treatment biocides were evaluated as bacterial water-soluble biocides. Each represented a major class of biocide type used conventionally in the water treatment industry. In addition, 2,4-dinitrophenol, a conventionally known bacterial water-soluble biocide was included in the test sequence for comparison. Details, as follows:

Table D

Test Substance	Code	Class of biocides

18

		represented
2,4 Dinitrophenol	A	-
Glutaraldehyde (15% active ingredient)	B	Aldehydes
Dodecyltrimethylammonium chloride (laboratory chemical)	C	Quaternary ammonium salts
Blend of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one (1.5% active ingredient)	D	Isothiazolones

5. Test Concentrations

Based on the liquid sludge, 2,4-dinitrophenol was dosed at 20 mg/l, this being
5   the known level at which significant uncoupling would result. The test biocides
were each evaluated at 1, 2.5, 5, 10 and 25 mg/l.

6. Procedure

10   Growth medium:

Glucose: 500 mg/l
Yeast extract Difco: 50 mg/l
Mineral nutrients: as described in Standard Method ISO 9888
15   (Determination of ultimate aerobic
                        biodegradability in aquatic environment)

Medium seeding:

20   50 mg/l homogenized and washed sample from municipal activated sludge
        treatment unit.

Incubation:

5   7 days at 20 °C in BOD-meter bottles (OxiTop)

The oxygen consumption was automatically determined and recorded all through the growth and decay phases (a total of 360 data points for each test substance).

10

Data was interpreted using a simple growth mathematical model as described above.

The determination of the model's coefficients are made through

15   adjustment and using the statistical test χ^2 to fit the model to the experimental data.

$$\chi^2 = \sum \frac{[OC_{observed} - OC_{theoretical}]^2}{OC_{theoretical}}$$

20   where OC is the oxygen consumption.

7. Results

Figure 4 summarises the results and shows that all three test biocides produced

25   significant and useful levels of uncoupling. Two of them (biocides C and D) produced greater levels of uncoupling at significantly lower dose levels than the conventional water-soluble biocide, 2,4-dinitrophenol. However, it should be borne in mind that these were screening tests and conditions were not optimised for the individual biocides and so it is possible that biocide B might have

30   produced greater uncoupling under more optimum conditions.

8. <u>Comments</u>

The results clearly show that representatives of three major water

5   treatment biocide classes will effect significant bacterial uncoupling at

low treatment levels.

10

15

20

25

30